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ORIGINAL ARTICLE

Recombinant foot-and-mouth disease virus (FMDV) non-structural protein 3A fused to enhanced green fluorescent protein (EGFP) as a candidate probe to identify FMDV-infected cattle in serosurveys

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Abstract Recombinant protein 3A-EGFP, a fusion construct between foot-and-mouth disease virus (FMDV) non-structural protein 3A and the enhanced green fluorescent protein (EGFP) was expressed in BL21-DE3 cells. The identity of the partially purified protein 3A-EGFP was confirmed by its reactivity with sera from cattle infected with FMDV and with a monoclonal antibody specific for FMDV-3ABC (MAb3H7) in Western blot assays. No reactivity was observed with sera from uninfected vaccinated animals. The performance of 3A-EGFP as an antigen in an indirect enzyme-linked immunosorbent assay (ELISA) was assessed and compared with that of a previously developed and validated capture ELISA that uses a 3ABC recombinant antigen (3ABC ELISA) and has been widely applied for serological surveys in Argentina. Parallel analysis of strongly and weakly positive reference sera from infected animals and 329 serum samples from uninfected vaccinated cattle showed that the 3A-EGFP antigen unequivocally identifies sera from FMDV-infected cattle with similar performance to its 3ABC counterpart. The 3A-EGFP ELISA is simpler and faster to perform than the 3ABC ELISA, since it does not require a capture step with a specific antibody. Moreover, the expression and storage of the recombinant 3A-EGFP is simplified by the absence of residual autoproteolytic activity associated to the 3C sequence. We conclude that the 3A-EGFP ELISA constitutes a promising screening method in serosurveys to determine whether or not animals are infected with FMDV.

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Introduction

The recognition and maintenance of the "foot-and-mouth disease virus (FMDV)-free with vaccination" status requires mandatory and reliable identification of FMDV-infected animals [1, 2]. This constitutes a major task for regulatory authorities in FMDV-free countries like Argentina, where a massive anti-FMDV vaccination program is applied [3]. FMDV non-structural proteins (NSPs) encoded by the P2 and P3 region of the genome are synthesized exclusively in FMDV-infected cells. The presence of antibodies against FMDV NSPs in cattle sera is therefore a clear indication of either past or present infection. FMDV-free vaccinated animals, on the other hand, are expected to show seroreactivity only against P1-derived capsid proteins present in commercial immunogens formulated with inactivated FMDV virions [4–7].

FMDV non-structural polypeptide 3ABC has been widely used as an antigen to detect FMDV-infection-induced antibodies in sera of suspected animals, irrespective of the vaccination status and of serotype involved [7–11]. Previously, a mutant 3ABC recombinant protein was produced [12] and used as an antigen in a two-step enzyme-linked immunosorbent assay (ELISA) test (3ABC ELISA) based on its capture by a monoclonal antibody specific for FMDV 3ABC (MAb3H7, [8]). This 3ABC ELISA was successfully applied to assist the regulatory authorities throughout the 2000–2002 FMDV outbreaks in Argentina and to support serosurveys to confirm the absence of viral

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infection. Alternative FMDV NSP proteins also derived from the FMDV P3 precursor (i.e., 3D, 3A, 3B and 3C) have been proposed as candidate antigens for use in ELISA tests and/or Western blots [5, 9, 12–16]. This report describes the expression of a fusion protein composed of FMDV 3A and EGFP [17] and explores its capacity as an antigen in a one-step ELISA, which does not require a specific capture antibody to differentiate sera from FMDV-infected and uninfected vaccinated cattle.

Materials and methods

Serum samples and reference panel

Sera labeled as A and B were derived from FMDV-convalescent cattle. Sera E and D were from animals that had no record of clinical FMD at the time of serum collection but tested positive by both 3ABC ELISA and ITB analysis [9], carried out in our laboratory and by the national authority (SENASA), respectively. The positive reactivity of these serum samples was confirmed using the OIE reference test (OIE manual de standard), showing strong reactivity (serum samples A and B) and close-to-cut-off reactivity (serum samples D and E). The weakly reactive sera D and E, represent sera that eventually require an alternative validation technique (i.e., immunoblotting) [9] to confirm the anti-FMDV-NSP activity. Serum samples F and G were obtained from uninfected vaccinated animals. Serum sample C was collected from a cow experimentally immunized with a recombinant 3ABC polyprotein expressed in bacteria as described [8, 12] and used to normalize RA values as described above. All sera used in this study were provided by the Servicio de Sanidad Animal (SENASA-Argentina). Table 1 shows corrected optical density (O.D.) readings from the seven reference cattle sera (A to G) that were used to calculate the RAs and standard errors (SE) from both ELISAs as shown in Figure 3.

Cloning and expression of FMDV 3A-EGFP

The genomic region encoding the 3A peptide (153 amino acids) of FMDV strain O1/Campos was cloned into the IPTG-inducible plasmid pET30a (Merck Millipore, USA) in frame with the EGFP gene amplified from the pEGFP-N1 plasmid (Takara Bio USA, USA). Briefly, the fragment containing the 3A gene (459 bp) was amplified from pET-3ABC (12) using the primers *p54a* (5' GACCATGGTCTCAATTCCTTCC-CAAAAATCTGTG 3') and *Rv.3A.EGFP* (5' CAGCTCCTCGCCCTTGCTCACCATTT-CAGCTTGTGGTTGCTCCTCAAC 3'). Simultaneously, the EGFP gene (720 bp) was amplified from pEGFP-N1 (Takara Bio USA, USA) using the primers *EGFP.Fw* (5' ATGGTGAGCAAGGGCGAGGAGCTGTTCACC 3') and *EGFP.Rv.HindIII* (5' CCAAGCTTTTACTTGTA-CAGCTCGTCCATGCCGAGAGTGATCCCGGCG 3'). Both products were subsequently used for an overlap PCR with the primers *p54a* and *GFP.Rv.HindIII*. Finally, the overlapped product was cloned into the NcoI and HindIII restriction sites of pET30a under the control of the phage T7 promoter (pET-3A-EGFP plasmid). The recombinant plasmid was sequenced to ensure the correct frame of the fused ORFs (Macrocen Inc, Korea). To obtain the FMDV 3A-EGFP peptide, competent *E. coli*.BL21 (DE3) cells [18] were transformed with the pET-3A-EGFP plasmid, and recombinant expression was induced with 1 mM IPTG at 37°C. Inclusion bodies were purified and solubilized in 6 M urea for use in immunoassays. Control recombinant EGFP (tagged with N-terminal GST) was obtained by inserting the GST ORF from pgstZ [19] into pEGFP-N1, using the HindIII and BamHI restriction sites.

3ABC ELISA

The 3ABC trapping ELISA was performed using a recombinant mutant 3ABC antigen (12) as described previously by Robiolo et al. [8]. Briefly, 96-well flat-bottom-well plates

Table 1 Optical density (O.D.) readings and their respective RA values for reference cattle sera A to G tested by both 3A-EGFP and 3ABC ELISA

Serum	3AGFP RA		3ABC RA		3AGFP O.D.		3 ABC O.D.	
	Me	(SE)	Me	(SE)	Me	(SE)	Me	(SE)
A	206.60	±17.28	90	±3.03	1.81	±0.03	1.34	±0.09
B	115.30	±11.40	70.20	±5.80	1.02	±0.04	1.05	±0.13
C	100	±1.20	100	±0.53	0.88	±0.02	1.48	±0.02
D	16.86	±2.27	19.64	±2.72	0.15	±0.01	0.29	±0.02
E	13.30	±1.62	13.86	±2.53	0.12	±0.01	0.20	±0.05
F	3.30	±1.42	1.70	±0.91	0.04	±0.01	0.02	±0.03
G	2.70	±0.50	1.03	±0.34	0.02	±0.01	0.02	±0.01

The mean (Me) and standard errors (SE) were obtained from eight independent determinations

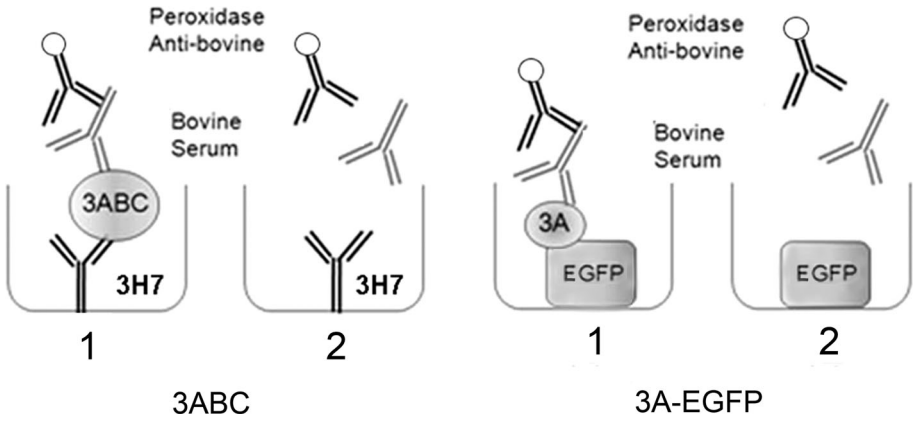

Fig. 1 3ABC ELISA and 3A-EGFP ELISA schemes. The 3ABC ELISA protocol requires coating wells with MAb 3H7, followed by incubation with either recombinant 3ABC- containing bacterial lysate (test well 1) or saline blocking buffer (control well 2). In the 3A-EGFP ELISA, test well 1 is coated with recombinant 3A-EGFP; and control well 2, with recombinant EGFP. Test serum is added to wells 1 and 2 in both ELISAs, followed by peroxidase conjugate and chromogenic development

(Nunc Maxisorb™) were coated overnight at 4°C with the mouse monoclonal antibody (MAb) 3H7 diluted in carbonate/bicarbonate buffer (pH 9.6) in a volume of 50 µl per well (Fig. 1). After four washes with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T0.05), 50 µl of 1:2000-diluted partially purified r3ABC (1 µg/µl) in dilution buffer (PBS-T containing 10% equine serum, PBS-T/E) was added to alternate rows of wells, incubated for 120 min at 37°C, and washed four times with PBS-T0.05. The assay was used as a single-dilution test. Duplicate serum samples diluted 1:40 in PBS-T/E were added to two different wells, one coated with the captured antigen (test well, Ag+) and the other without antigen (control well, Ag−). After incubation for 30 min at 37 °C and four washings, 50 µl of 1:5000-diluted horseradish-peroxidase-conjugated goat anti-bovine serum (Jackson ImmunoResearch Laboratories Inc., Pennsylvania) diluted in PBS-T/E was added and incubated for 30 min at 37 °C. Finally, color was developed using the chromogen/substrate mixture ABTS/H_2O_2 (ABTS: 2, 2-azinobis [3-ethylbemziazoline-6-sulfonic acid]) and stopped after 30 min by the addition of 50 µl of 0.2% NaF. The absorbance in each well was read at 415 nm. The optical density (O.D.) for each sample was calculated by subtracting the O.D. value of the Ag- (antigen negative) well from that of the corresponding Ag+ (antigen positive) well.

3A-EGFP ELISA

Ninety-six-well flat-bottom-well plates (Nunc Maxisorb™) were first coated overnight at 4°C with 50 µl of partially purified 3A-EGFP (1 ng/µl in carbonate/bicarbonate buffer, pH 9.6), in alternate rows of wells (Ag+, test wells, see Figure 1). The remaining wells were coated with the same amount of GST-EGFP under the same conditions (control wells, Ag-). For blocking, all wells were incubated 60 min in PST-T (PBS-0.05M Tris buffer containing 10% equine serum and 3% non-fat milk) and washed four times with PBS-T. The assay was used as a single-dilution test. Duplicate serum samples diluted 1:100 in PBS-T/E were independently added to pairs of wells (Ag+ and Ag-) and incubated for 60 min at 37°C. After four washes with PBS-T0.05, 50 µl of 1:5000-diulted horseradish-peroxidase-conjugated goat anti-bovine serum (Jackson ImmunoResearch Laboratories Inc., Pennsylvania) diluted in BST-T was added and incubated for 60 min at 37°C. Finally, after four washings, color was developed using the chromogen/substrate mixture ABTS/H_2O_2 (ABTS: 2, 2-azinobis [3-ethylbemziazoline-6-sulfonic acid]) and stopped after 30 min by the addition of 50 µl of 0.2% NaF. The absorbance in each well was read at 415 nm or 405 nm. The corrected O.D. for each sample was calculated by subtracting the O.D. value of the Ag- well from that of the corresponding Ag+ well.

The relative activity (RA) of each individual serum, for the 3A-EGFP and 3ABC ELISAs was calculated by dividing the corrected O.D. values (test wells minus control wells) by the corrected O.D. value of the reference serum C and expressed as a percentage. Serum C was obtained from a cow immunized with recombinant 3ABC protein, and O.D. values obtained with this serum were considered 100% reactive and used for normalization in both ELISAs. Table 1 shows the optical density readings and their respective RAs (shown in Fig. 3B) for each serum of the reference panel (sera A to G) obtained with both ELISAs.

Western blot assay

Samples were resolved by 12% SDS-PAGE, transferred to a Hybond® ECL nitrocellulose membrane (GE Healthcare) and blocked overnight in 5% PBS-TM (PBS, 0.1% Tween 20 [PBS-T0.1] supplemented with 5% non-fat milk). The membrane was probed with rabbit anti-GFP (1:2500, GFP

Tag Polyclonal Antibody, Invitrogen), mouse anti-3A MAb3 H7 or bovine serum samples (1:50) for 60 min at room temperature. Bovine serum samples were pre-incubated overnight with 50 ng of EGFP-GST inclusion bodies in 1 ml of PBS-TM. The membrane was then washed three times for 5 minutes in PBS-T0.1, and then probed with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, 1:5000), anti-mouse IgG HRP conjugate (Promega, 1:10000), or horseradish-peroxidase-conjugated goat anti-bovine serum (1:5000, Jackson ImmunoResearch Laboratories Inc., Pennsylvania) in 3% PBS-TM for 1 h at room temperature. Membranes were washed three times in PBS-T0.1, and antibody binding was detected using an ECL chemiluminescence (Pierce Western Detection Reagent®) in a G:Box (Chemi XRQ, Syngene).

Statistical analysis

ELISA data processing was carried out using ANOVA and Prism 5® software (v5.03. Graph Pad Software, Inc.).

Results

The DNA fragments encoding protein 3A of FMDV strain O1/Campos and the EGFP protein were cloned in frame (Fig. 2A) into plasmid pET30a and expressed in transformed BL21-DE3 cells upon induction with IPTG. Partially purified inclusion bodies were solubilized in 6 M urea and analyzed by SDS-PAGE followed by immunoblotting and independent incubation with cattle sera and specific antibodies. The Western blot profile in Figure 2B confirms the expression of a fusion protein (3A-EGFP) with the expected molecular weight (44-45 kDa) that reacted with both specific anti-3ABC monoclonal MAb H7 and rabbit anti-GFP polyclonal serum. Protein 3A-EGFP reacted strongly with serum from FMDV-infected cattle (lane 2, A serum), but no reactivity was detected with serum from a FMDV-free vaccinated animal (lane 2, G serum). The excellent signal-to-background ratio shown in the Western blot profile suggested that 3A-EGFP could be an appropriate candidate as a seroprobe in an immunoassay to distinguish sera from FMDV-infected and uninfected animals, irrespective of their vaccination status.

We tested the reactivity of 3A-EGFP in an indirect ELISA against a panel of seven reference sera (A to G). Comparative performance with the currently used 3ABC ELISA was evaluated by running both assays in parallel with the same reference sera. The bar graph in Figure 3A shows the relative immunoreactivity with respect to reference serum C (100% RA, see also Table 1) for sera of the reference panel assayed in both 3A-EGFP and 3ABC ELISAs in eight independent determinations carried out in duplicate on different days. As can be seen in Figure 3B,

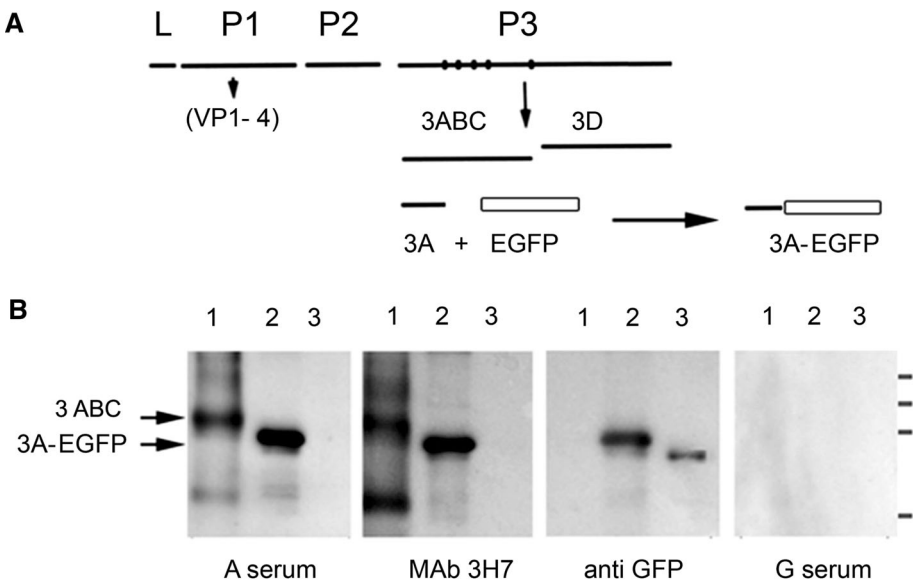

Fig. 2 A) FMDV- P3 precursor processing. The FMDV primary translation product is processed into L, P1, P2 and P3 (or 3ABCD) polypeptides. Alternative cleavage at the 3C-3D junction generates polypeptides 3ABC and 3D, the viral RNA polymerase. The 153-amino-acid-long protein 3A is derived from the N-terminus of 3ABC. Green fluorescent protein sequences are fused to the C-terminus of FMDV 3A, and the hybrid protein (3A-EGFP) is expressed in *E. coli* BL21 (DE3) bacteria. B) Partially purified inclusion bodies from cells expressing recombinants 3ABC (lane 1), 3A-EGFP (lane 2), and GST-EGFP (lane 3) were separated by 12% SDS-PAGE, blotted to a nitrocellulose membrane, and tested against reference sera from convalescent (A serum) and vaccinated (G serum) cattle. Controls with anti-GFP polyclonal serum and MAb 3H7 were also included as references. Bars on the right represent molecular weight markers (100, 70, 55 and 35 kDa, from top to bottom)

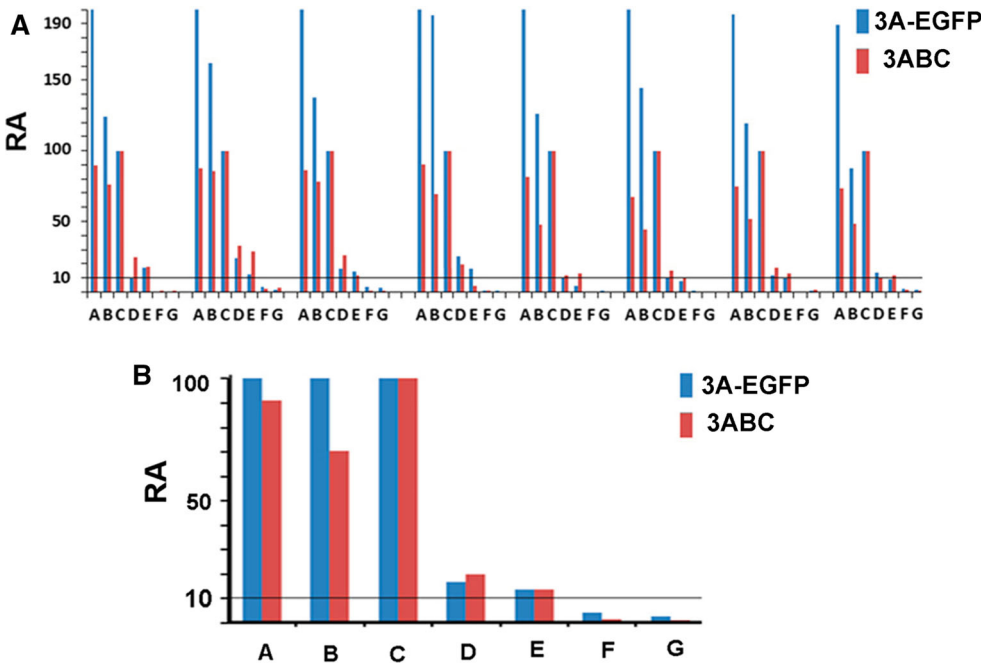

Fig. 3 A) Bar graph representing RA values obtained in eight independent determinations with certified positive sera (A, B and C), weakly reactive sera (D and E), and negative controls from FMDV-free vaccinated animals (F and G) with both ELISAs. Values are the average of duplicate readings corrected according to their respective controls. B) Bar graph representing mean RA values on a 0 to 100% scale (see also Table 1). The 10% threshold is shown by a horizontal line in both graphs

RAs for positive sera A and B were clearly differentiated from the background values of negative sera F and G in both 3A-EGFP ELISA (≈207 and 115% vs. 3.3 and 2.7%, respectively) and 3ABC-ELISA (≈90 and 70.2 % vs. 1.70 and 1. 03%, respectively). The mean RA values for the weakly positive sera D and E fell within the 10-20% range in both ELISAs (≈16.9 and 13.3% vs. 19.6 and 13.9 %, respectively) and were 5- to 10-fold higher than those from negative sera F and G. The 3A-EGFP-based ELISA was therefore capable of detecting anti-FMDV activity in reference sera A, B and C and seems to have been as sensitive as the 3ABC ELISA in its ability to distinguish the weakly reactive sera D and E from the negative FMDV-free serum controls.

We further tested the reactivity of the weakly positive and negative sera in Western blot assays [9] in combination with the highly sensitive chemiluminiscence method for detection. Figure 4 shows the reactivity profile of sera D and E against recombinant antigens 3A-EGFP and EGFP (lanes 1 and 2, respectively) blotted onto nitrocellulose. Positive and negative controls (sera A and G, respectively) along with MAb 3H7 [8] and rabbit polyclonal anti-GFP serum, were included as references. The results showed high reactivity of the positive control (serum A) with recombinant 3A-EGFP, but not with the recombinant EGFP protein. Weakly reactive bands corresponding to protein 3A-EGFP were detected (see arrows) in the blot incubated with sera D and E, but not in the corresponding blot incubated with negative control (G serum) under identical conditions of exposure and signal development. These results confirm the presence of a weak but specific anti-FMDV-3A activity in sera D and E and are compatible with the data shown previously with both ELISAs.

A preliminary assessment of the specificity of the 3A-EGFP test was based on calculating the RA values of 329 serum samples from uninfected vaccinated animals. The bar graph in Figure 5 shows serum RA profiles obtained with the 3A-EGFP ELISA and in parallel with the 3ABC ELISA for comparison (blue and red bars, respectively). Also shown for reference are bars representing individual and mean RA values from eight independent determinations with D and E sera (previously shown in Fig. 3), as well as the mean values (Vm) for the total of 329 determinations from the 3A-EGFP and 3ABC ELISAs. All but two (99%) and 100% of the total of 329 sera from FMDV-free vaccinated cattle showed RAs below the 10% RA threshold when tested with the 3A-EGFP ELISA and the 3ABC ELISA, respectively. The mean RA value obtained with the 329 samples tested with 3A-EGFP and 3ABC ELISAs were 2.13 and 1.52 %, respectively. They were at least 5-fold lower than the mean RA values for the weakly positive sera D and E, which fell consistently within the 10-20% range (see also Fig. 3B). Most of the 329 sera tested from vaccinated animals showed RAs of 5% or lower (92% and 97% for the 3A-EGFP and 3ABC ELISA, respectively). The results suggest that the specificity of the 3A-EGFP ELISA is equivalent to than the 3ABC ELISA and can be used to screen sera from FMDV-free vaccinated animals.

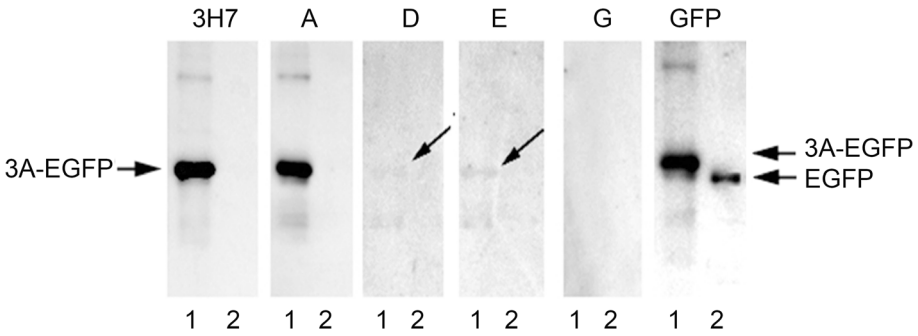

Fig. 4 Reactivity of cattle sera D and E in Western blot. Recombinant antigens 3A-EGFP (lane 1) and EGFP (lane 2) were tested against positive reference serum A and a negative control from FMDV-free vaccinated cattle (serum G) as well as with weakly positive sera D and E. MAb 3H7 and anti GFP antibodies were included for reference. Arrows point to weakly reactive 3A-EGFP bands detected by sera D and E, but not by the negative serum control G

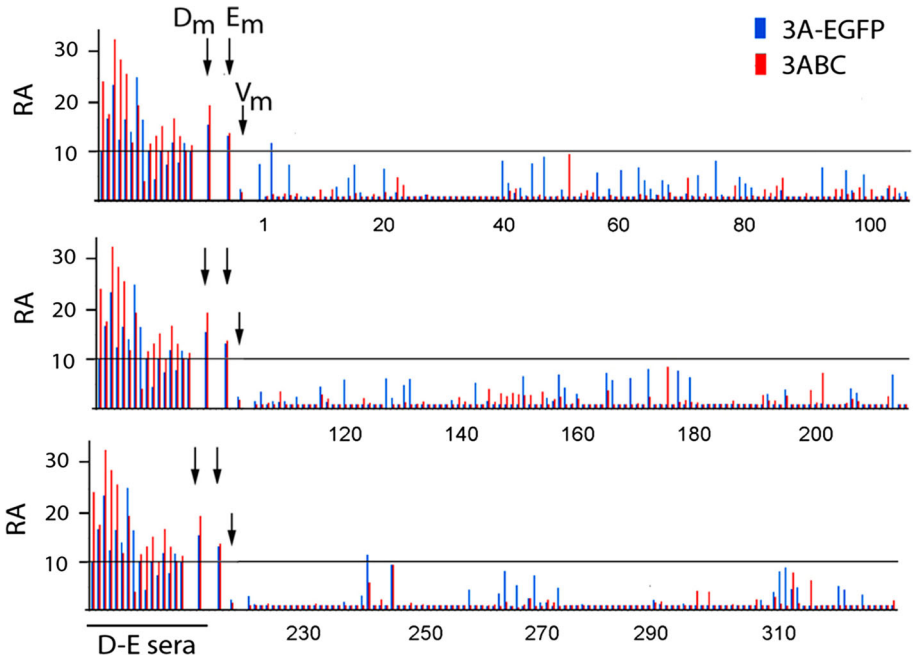

Fig. 5 Immunoreactivity profile of sera from 329 FMDV-free (uninfected) vaccinated cattle. Blue and red bars represent individual serum RA values obtained with 3A-EGFP and 3ABC ELISAs, respectively. "D-E sera" indicates RA values of each of the eight independent assays, and arrows labeled Dm and Em to their respective mean values. Mean RA values (Vm) of the total of 329 samples tested with 3A-EGFP and 3ABC ELISAs were 2, 13 and 1.52%, respectively, and their corresponding standard errors were ±0.90 and ±0.40%. The line at 10% RA represents the tentative cutoff value for screening FMDV-free negative sera. Individual sera were tested in duplicate as described in Materials and methods

Discussion

We have previously reported on the performance of the 3ABC ELISA and its application in large-scale screening serosurveys to confirm the absence of viral activity in FMDV-vaccinated cattle herds [8]. The test has been successfully used in FMDV serosurveys by the National Health Authority in Argentina and quality control of commercial FMDV vaccines, (i.e., evaluation of anti-FMDV-NSP immunity induced by vaccination).

The 3ABC ELISA is a multi-step protocol that requires overnight coating of multiwell plates with capture MAb 3H7 followed by incubation with a 3ABC-expressing bacterial lysate, addition of test serum, and developing of the chromogenic signal. The capture is meant to minimize the presence of bacterial contaminants reacting with non-specific antibodies and reducing the signal-to-noise ratio of the assay. The protocol also requires correction of individual test serum readings against captured 3ABC (test well) by subtracting those from parallel control wells preincubated with saline buffer (see Fig. 1).

The relatively complex format of the 3ABC ELISA encouraged us to test an alternative antigen in a simpler one-step, ELISA format that is able to provide a more economical, rapid and easier throughput screening method. It had already been shown that proteins other than 3ABC and those derived from the P3 region of the FMDV genome, 3A among them, are reactive with sera from convalescent cattle and hold potential as candidates for FMDV serosurveys [13–16]. The association of FMDV 3A with an EGFP carrier was originally meant to decrease its relative mobility in SDS PAGE analysis and simplify its identification and eventual chromatographic purification. EGFP is a protein with a complex folding pattern [17] and Yantsevich *et al*. [20] have suggested that it is likely to contribute to the conformation stability of associated heterologous C- or N-terminal fusion sequences.

Analysis of 329 serum samples collected from vaccinated animals in both the 3A-EGFP and the 3ABC ELISAs showed that almost all RA values were below the 10% threshold. Repeated testing of weakly reactive positive sera showed RA values within the 10-15% range, 5- to 10-fold higher than those from negative controls and the mean RA value from the 329 vaccinated cattle. Taken together, the results suggest that an RA of 10% could be an appropriate cutoff value to identify FMDV-free vaccinated animals when using 3A-EGFP in serosurveys.

A wider range between positive to weak positive reactivity could be established for the 3A-EGFP ELISA when compared with the 3ABC ELISA. Indeed, whereas similar values were obtained for weakly positive sera in both tests, ELISA RA values for the strongly positive sera A and B obtained with the 3A-EGFP ELISA reached 206.6% and 115.3 %, respectively, which were considerably higher than those obtained with the 3ABC ELISA (90.4% and 70.2%, respectively). Whether this is related to a major anti-FMDV-3A component among the anti-FMDV-NSP antibodies present in sera of convalescent infected cattle remains to be investigated. It is worth noting, however, that the capture monoclonal antibody MAb 3H7 maps to the first 30 N-terminal amino acids of 3ABC, within the 3A sequence (unpublished observation). The weaker signals obtained in the the 3ABC ELISA could be thus explained by possible interference with a significant fraction of the anti-3A antibodies present in samples from FMDV-infected animals.

The 3A-EGFP recombinant is recovered in larger proportions than its mutant 3ABC counterpart according to SDS-PAGE analysis of bacterial inclusion bodies purified after IPTG induction (not shown). This difference, most likely explained by the absence of a 3C-associated residual autoproteolytic activity, constitutes a major advantage for long-term storage of the 3A-EGFP recombinant and its eventual use in a ready-on-demand antigen-coated multiwell plates. Further validation of the 3A-EGFP-based ELISA will be pursued, as well as the potential of using a dual system of serodiagnosis based in a simple and faster 3A-EGFP ELISA for initial screening followed by confirmation of positive or suspect samples with the already validated 3ABC ELISA.

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Compliance with ethical standards

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Conflict of interest C M. Lotufo declares that she has no conflict of interest. I.E. Bergmann declares that she has no conflict of interest. N.M. Mattion declares that she has no conflict of interest. M. Wilda declares that he has no conflict of interest. P.R. Grigera declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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